CHARACTERIZATION AND OCHRATOXIN 'A' PRODUCTION OF *Aspergillus* SECTION *Nigri*

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CHARACTERIZATION AND OCHRATOXIN 'A' PRODUCTION OF *Aspergillus* SECTION *Nigri*

by

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TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

7 d	Seven days
ACP	Acyl carrier protein
AFLP	Amplified Fragment Length Polymorphisms
AFs	Aflatoxins
AT	Acyl transferase
a_w	Water activity
BEN	Balkan Endemic Nephropathy
BLAST	Basic Local Alignment Search Tool
CBS	Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre
C-Met	C-methyltransferase
CREA	Creatine Sucrose Agar
CY20S	CYA with 20% sucrose
CYA	Czapek Yeast Extract Agar
CZ	Czapek's Solution Agar
DG18	Dicholoran 18% Glycerol Agar
DH	Dehydratase
DNA	Deoxyribonucleic Acid
dNTPs	Dinucleotide triphosphates
ELISA	Enzyme-linked Immunosorbent Assay
ER	Enoylreductase
EtBr	Ethidium bromide
FB_2	Fumonisin B2
FLD	Fluorescence detector
GRAS	Generally Regarded As Safe
HAL	Halogenase
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ICBN	International Code of Botanical Nomenclature
ICPA	International Commission on *Penicillium* and *Aspergillus*
ITS	Internal transcribed spacer
ISHAM	International Society of Human and Animal Mycology

KR	Ketoreductase
KS	β-ketoacyl synthase
LPCB	Lactophenol Cotton Blue
MEA	Malt Extract Agar
MEA20S	MEA with 20% sucrose
MEGA	Molecular Evolution and Genetic Analysis
ML	Maximum Likelihood
MY40G	Malt Yeast 40% Glucose Agar
NCBI	National Centre for Biotechnology Information
NJ	Neighbour Joining
NRPS	Nonribosomal peptide synthase
NTSYS	Numerical Taxonomy and Multivariate Analysis System
OA	Oatmeal Agar
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
OTα	Ochratoxin α
OTβ	Ochratoxin β
P450	Cytochrome P450 monooxygenase
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragmenth Length Polymorphism
PKS	Polyketide synthase
RAPD	Random Amplified Polymorhic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RPB2	RNA polymerase II second largest subunit
rpm	Revolutions per minute
SEM	Scanning Electron Microscope
SNPs	Single-nucleotide polymorphisms
sp.	Species
TBE	Tris Borate EDTA
TEF	Translation elongation factor-alpha
TF	bZIP transcription factor

TLC	Thin Layer Chromatography
UHPLC-FLR	Ultra-High Performance Liquid Chromatography
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet
WA	Water Agar
YES	Yeast Extract Sucrose agar

PENCIRIAN DAN PENGHASILAN OKRATOKSIN 'A'

OLEH *Aspergillus* SEKSYEN *Nigri*

ABSTRAK

Beberapa spesies *Aspergillus* hitam atau *Aspergillus* seksyen *Nigri* boleh bertindak sebagai saprofit serta bahan pencemar makanan dan makanan ternakan. Terdapat juga *Aspergillus* hitam yang toksigenik, menghasilkan okratoksin A (OTA) yang merupakan antara mikotoksin diketahui berasosiasi dengan makanan dan makanan ternakan. Toksin ini boleh mengakibatkan kesan buruk kepada manusia dan haiwan. Tujuan kajian ini adalah untuk mengenal pasti dan menciri spesies *Aspergillus* hitam daripada pelbagai substrat dan persekitaran dalaman di Malaysia, menentukan hubungan filogenetik di antara spesies, menciri spesies menggunakan teknik pembatasan kepanjangan pecahan polimorfisme (RFLP) pada jujukan kawasan penjarak transkipsi dalaman (ITS) dan menentukan kebolehan spesies ini menghasilkan OTA. Dalam kajian ini, 177 pencilan *Aspergillus* hitam daripada makanan (beras, kacang tanah dan rempah), makanan ternakan (bijirin jagung), tanah dan persekitaran dalaman telah dikenal pasti secara morfologi sebagai *A. niger* (153 pencilan) dan *A. aculeatus* (24 pencilan). Berdasarkan jujukan ITS, β-tubulin dan kalmodulin, spesies telah dikenal pasti semula sebagai *A. niger* (144 pencilan), *A. aculeatus* (24 pencilan) dan *A. tubingensis* (sembilan pencilan). Analisis filogenetik jujukan ITS, β-tubulin and kalmodulin secara individu dan gabungan menggunakan kaedah hubungan jiran dan kebolehjadian maksimum menunjukkan *A. niger*, *A. aculeatus* dan *A. tubingensis* telah dikelompokkan mengikut spesies. Teknik pembatasan kepanjangan pecahan polimorfisme ITS telah dijalankan untuk mencirikan lagi pencilan *Aspergillus* hitam. Corak jalur pembatasan *Rsa*I dapat

membezakan *A. niger*, *A. aculeatus* dan *A. tubingen*sis, berdasarkan tiga jenis corak jalur. Corak jalur pembatasan *Hinf*I, *Hin*dIII (*Nla*III), *Hha*I dan *Taq*I dapat membezakan *A. aculeatus* daripada *A. niger* dan *A. tubingensis* tetapi tidak dapat membezakan antara *A. niger* dan *A. tubingensis.* Analisis kluster UPGMA pada jalur pembatasan ITS adalah selari dengan pengenalpastian secara molekul yang mana pencilan daripada spesies yang sama dikelompokkan dalam klad yang sama. Gen OTA telah dikesan hanya pada enam pencilan *A. niger* daripada beras (tiga pencilan) dan persekitaran dalaman (tiga pencilan). Berdasarkan analisis kromatografi cecair berprestasi ultra tinggi dipasang dengan pendarfluor, pencilan tersebut menghasilkan pelbagai kepekatan OTA, berjulat dari 0.26 sehingga 3.26 µg/g. Sebagai kesimpulan, tiga spesies *Aspergillus* hitam telah dikenal pasti di dalam kajian ini iaitu *A. niger* (81.36%), *A. aculeatus* (13.56%) and *A. tubingensis* (5.08%). Kejadian *A. niger* yang tinggi menunjukkan bahawa spesies ini mudah dijumpai di iklim tropika sperti Malaysia. Walau bagaimanapun, *A. niger* yang okratoksigenik bukanlah kelaziman di Malaysia memandangkan hanya enam pencilan didapati menghasilkan OTA pada tahap kepekatan yang rendah.

CHARACTERIZATION AND OCHRATOXIN 'A' PRODUCTION OF *Aspergillus* SECTION *Nigri*

ABSTRACT

Several species of black *Aspergillus* or *Aspergillus* section *Nigri* can act as saprophytes as well as contaminants of food and feed. There are also black *Aspergillus* that are toxigenic, producing ochratoxin A (OTA) which is a mycotoxin known to be associated with food and feed. This toxin can cause adverse effects to humans and animals. The aims of this present study were to accurately identified and characterize black *Aspergillus* from various substrates and indoor environment in Malaysia, to determine the phylogenetic relationships between the species, to characterize the species using Restriction Fragment Length Polymorphism (RFLP) technique of internal transcribed spacer regions (ITS) and to determine the ability of the species to produce OTA. In this study, 177 isolates of black *Aspergillus* from food (rice, groundnuts and spices), feed (corn grains), soil and indoor environment were morphologically identified as *A. niger* (153 isolates) and *A. aculeatus* (24 isolates). Based on sequences of ITS, β-tubulin and calmodulin, the species were re-identified as *A. niger* (144 isolates), *A. aculeatus* (24 isolates) and *A. tubingensis* (nine isolates). Phylogenetic analysis of individual and combined ITS, β-tubulin and calmodulin sequences using Neighbour-joining and Maximum Likelihood methods showed that *A. niger*, *A. aculeatus* and *A. tubingensis* were clustered according to the species. Restriction Fragment Length Polymorphism technique of ITS was carried out to further characterize the black *Aspergillus* isolates. Restriction patterns of *Rsa*I were able to differentiate *A. niger*, *A. aculeatus* and *A. tubingensis*, based on three types of banding patterns. Restriction patterns of *Hinf*I, *Hin*dIII (*Nla*III), *Hha*I and

*Taq*I were able to differentiate *A. aculeatus* from *A. niger* and *A. tubingensis* but cannot distinguished between *A. niger* and *A. tubingensis*. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of ITS restriction bands was in accordance with molecular identification of which the isolates of the same species were clustered in the same cluster. OTA gene was detected in only six isolates of *A. niger* from rice (three isolates) and indoor environment (three isolates). Based on Ultra-High Performance Liquid Chromatography coupled with fluorescence analysis, the isolates produced varying concentrations of OTA, ranging from 0.26 to 3.26 µg/g. As a conclusion, three black *Aspergillus* species were identified in this study that is, *A. niger* (81.36%), *A. aculeatus* (13.56%) and *A. tubingensis* (5.08%). High occurrence of *A. niger* showed that this species are ubiquitous and easily found in tropical climate such as Malaysia. However, the ochratoxigenic *A. niger* is not prevalent in Malaysia as only six isolates were found producing OTA with low level of concentrations.

CHAPTER 1

INTRODUCTION

Aspergillus is a very large genus of spore-forming fungi distributed worldwide. *Aspergillus* spp. are able to grow on various substrates and cause degradation of agricultural products both before and after harvest (Klich, 2002a). Hence, *Aspergillus* spp. are common food and feed spoilage as well as common soil and indoor air fungi. One of the most important sections of *Aspergillus* spp. is section *Nigri* or is known as black *Aspergillus*. Members of black *Aspergillus* such as *A. niger, A. tubingensis* and *A. carbonarius* occurred naturally on many substrates including various types of food and feed, different types of soils and diverse indoor environments. As black *Aspergillus* species have a cosmopolitan distribution, these species can also be widely found in the tropics, mainly related to hot and humid climate.

Black *Aspergillus* can act as toxigenic fungi and is associated with production of ochratoxin A (OTA) which is one of the most common mycotoxins detected in food and feed. Ochratoxin A has been reported in many types of food and feed worldwide either detected in the substrates or produced by the ochratoxigenic strains of black *Aspergillus* (Leong et al., 2007; Ponsone et al., 2007; Chiotta et al., 2010; Moslem et al., 2010; Kittikamhaeng and Dachoupakan, 2011; Storari et al., 2012; Lasram et al., 2013). Ochratoxin A contamination by black *Aspergillus* might cause a potential risk on animal health and food safety as this mycotoxin can be transferred through the food chain. In a large scale, the contamination of OTA can also cause economic losses to livestock and agricultural products. The most common

ochratoxigenic black *Aspergillus* species are *A. carbonarius* and *A. niger*. Ochratoxigenic strains of these two species of black *Aspergillus* might also occurred in Malaysia due to tropical climate with high relative humidity and temperature, that are suitable for the production of OTA.

In Malaysia, black *Aspergillus* is usually referred to as *A. niger* although black *Aspergillus* comprises many species. This is primarily due to identification that was based solely on morphological characteristics. Moreover, black *Aspergillus* consisted of closely related species that have similar morphological characteristics, and several researches regarded black *Aspergillus* as a species complex or species aggregate due to overlapping morphological characteristics (Balajee et al., 2007). If molecular method was used, only Internal Transcribed Spacer (ITS region) was applied which is not sufficient as the region can only be used for rough classification of uniseriate and biseriate species (Samson et al. 2007). Therefore, secondary markers are needed in combination with ITS region for phylogenetic analysis and to strengthen the identification of black *Aspergillus* into species level.

Due to the potential risk of black *Aspergillus* as OTA producer, it is very important to identify them accurately as there might be more than one species of black *Aspergillus* occurred in Malaysia. This study was conducted to identify species of black *Aspergillus* in various or different substrates as well as in indoor environment and to determine their ability to produce OTA as *Aspergillus* is cosmopolitan species and thrive in tropical climate. Various substrates including food and feed such as ice, groundnuts, spices and corn grains as well as soils were chosen as these substrates support good growth of black *Aspergillus.* Groundnuts and spices are food commodities widely used in Malaysia and corn grains are widely used as livestock feed. Black *Aspergillus* are common soil fungi associated with

decomposition of plant materials and some species are plant pathogens (Hong et al., 2013; Kocsube et al., 2013). Species of black *Aspergillus* are also frequently encountered in indoor environment (Wardah et al., 2012; Varga et al., 2014; Ghosh et al., 2014) and several species were able to produce OTA (Samson et al., 2007). Although, black *Aspergillus* isolates are common in Malaysia, the species composition is not well-documented. From this study, information on the occurrence of common species of black *Aspergillus* as well as ochratoxigenic isolates will assist in the development of suitable strategies in minimizing the risk of OTA contamination in foods, feed as well as in indoor environment.

In order to correctly identified *Aspergillus* spp. particularly black *Aspergillus*, a polyphasic approach as recommended by Samson et al. (2014) is applied. Polyphasic approach is a combination of several approaches including morphology, extrolite, molecular and phylogenetic data that are used for identification. The first step for identification of *Aspergillus* spp. is based on morphological characteristics which include the colony appearance, pigmentation and textures, colony diameters, shapes of conidial heads, formation of phialides, and size and texture of conidia and vesicle (Klich, 2002b; Pitt and Hocking, 2009; Samson et al., 2010). The most important characters are the colony appearance, formation of phialides and the characteristics of conidia. Morphological identification is used to sort the isolates into sections or tentatively identified the isolates to species levels. However, the used of morphological characteristics for identification of black *Aspergillus* has limitation as some of the characters could not provide a clear separation of species as most of the species within this section have similar morphological features. Thus, molecular identification and phylogenetic analysis are carried out to support morphological identification and re-confirm the species identity.

For molecular identification and characterization, several regions or genes are used. Combinations of more than one region or genes are applied to confidently identify the species. For *Aspergillus* spp., internal transcribed spacer (ITS) region is regarded as primary marker and commonly used for identification and characterization to species level. The ITS is a non-coding regions, easy to amplify and a large number of reference sequences exist in the GenBank (Balajee et al., 2009; Samson et al., 2010). However, several closely related species of black *Aspergillus* produced identical ITS sequences and therefore, protein coding genes including β-tubulin and calmodulin genes are used as secondary markers to provide sufficient nucleotides differences to distinguish species of black *Aspergillus* (Samson et al., 2007).

After molecular identification, phylogenetic analysis is conducted as this approach is very useful in defining species and classifying the taxa of *Aspergillus* spp. Phylogenetic analysis is a comparative sequence analysis for studying the genetic relationship of a group of organisms. In this study, phylogenetic analysis of individual and combined sequences of ITS region, β-tubulin and calmodulin genes are carried out to group black *Aspergillus* into several clades as well as to re-confirm the species. The used of β-tubulin gene for phylogenetic analysis combined with ITS region and calmodulin gene for identification of species are recommended by Samson et al. (2010) and Samson et al. (2014).

Another method used for characterization of *Aspergillus* spp. is a combination of PCR and Restriction Fragment Length Polymorphism (PCR-RFLP). This method was proposed as one of the fast and easy method to be used especially in studies that involve a large number of isolates (Martinez-Culebras and Ramón, 2007). The ITS region has been applied in PCR-RFLP analysis as this region

contains high degree of sequence polymorphism that is suitable for intraspecies sequence diversity. The ITS-RFLP is also useful in characterization of closely related species of black *Aspergillus* such as to differentiate between *A. niger* and *A. tubingensis* (Accensi et al., 1999; Medina et al., 2005).

Production of secondary metabolites occasionally is adequate to be used in identification of toxigenic *Aspergillus* spp. (Samson et al., 2014). For black *Aspergillus,* the production of OTA can be used to characterize ochratoxigenic *A. niger* and *A. carbonarius.* To detect OTA producers of black *Aspergillus*, PCR amplification using specific primers of OTA biosynthetic gene cluster, the polyketide synthase (PKS) gene, is commonly used (Kim et al., 2014). Detection of PKS gene is important for a fast and early detection of the ability of black *Aspergillus* isolates to produce OTA. After the PKS gene has been detected, the production and quantification of OTA is carried out using analytical method including Ultra-High Performance Liquid Chromatography coupled with fluorescence (UHPLC-FLR).

As black *Aspergillus* are easily spread and contaminated various foods and feeds that may lead to potential toxigenic risk to human and animal, it is essential to accurately identify species of black *Aspergillus* from different substrates and indoor environment to determine the mycotoxigenic strains/species. Therefore, the specific objectives of this study were:

1) To identify and characterize black *Aspergillus* from various sources including food, feed, soil and indoor environment based on morphological and molecular characteristics,

2) To determine phylogenetic relationships of black *Aspergillus* using ITS region, β-tubulin and calmodulin genes,

3) To characterize black *Aspergillus* using PCR-RFLP analysis of ITS region,

4) To detect OTA biosynthetic gene cluster and to quantify OTA production by black *Aspergillus* species using UHPLC-FLR.

CHAPTER 2

LITERATURE REVIEW

2.1 History of *Aspergillus* Taxonomy

The genus *Aspergillus* was first introduced by a Florentine priest-mycologist, P. A. Micheli in 1729 based on the spore-bearing structure that resembled an aspergillum, a brush-like structure used in religious ceremonies to sprinkle holy water (Raper and Fennell, 1965; Palencia et al., 2010). Micheli then used *Aspergillus* and *Mucor* spp. as examples of asexual reproducing fungi in the description of spore formation (Baker and Bennett, 2007).

The first monograph of *Aspergillus*, 'The Aspergilli' which contained a large taxonomic literature and description of *Aspergillus* cultures under controlled condition was published by Thom and Church (1926). In the monograph, 69 species were accepted and placed in 11 groups. Later, 'A Manual of the Aspergilli' was published by Thom and Raper (1945) which recognized 77 species, eight varieties and four mutations. The mutant cultures were derived by ultraviolet irradiation of wild strain of *A. fonsecaeus*, a species in *A. niger* group (Vezina and Raper, 1957). Another monograph, 'The Genus *Aspergillus*' was published by Raper and Fennell (1965) that contain description of 132 species and 18 varieties, classified into 18 groups according to similar physiological or biochemical activities. The descriptions included species of black *Aspergillus* which were recognized by brown to black-shaded spores and were placed in '*Aspergillus niger* group'.

Samson (1979) provided a compilation and discussion of 90 new *Aspergillus* species and varieties, since Raper and Fennell (1965). Earlier species descriptions of *Aspergillus* based on descriptions by Thom and Church (1926), Thom and Raper (1945) and Raper and Fennell (1965) contained information on the teleomorph and anamorphic genera but the description did not follow the International Code of Botanical Nomenclature (ICBN) guidelines. Therefore, the teleomorphic and anamorphic *Aspergillus* spp. accepted by Raper and Fennel (1965) were further typified and revised by Samson and Gams (1985), Gams and Samson (1985) and Gams et al. (1985). Gams and Samson (1985) provided typification of *Aspergillus* and its associated teleomorphs while Samson and Gams (1985) typified the species of *Aspergillus* accepted by Raper and Fennell (1965).

At that time, the use of the term 'group' was abandoned and formally changed to subgenera and sections as proposed by Gams et al. (1985). Gams et al. (1985) divided *Aspergillus* and its teleomorphs into six subgenera and 18 sections that corresponded to 18 groups described by Raper and Fennell (1965). For example, species of black *Aspergillus* were included in subgenus *Circumdati* and section *Nigri*. The use of subgenera and sections are accepted by the International Commission on *Penicillium* and *Aspergillus* (ICPA).

Through phylogenetic analysis based on D1 and D2 regions of large subunit rDNA, Peterson (2000) proposed to eliminate three subgenera, *Ornati, Clavati* and *Circumdati* of the six subgenera by Gams *et al.* (1985) and retained only 12 sections (*Aspergillus, Restricti, Fumigati, Cervini, Ornati, Clavati, Nidulantes, Flavi, Nigri, Circumdati, Candidi* and *Cremei*) of the 18 sections, Peterson (2000) also modified three sections (*Terrei, Flavipedes* and *Sparsi*) and eliminated three sections (*Versicolores, Usti* and *Wentii*). In the same year, Pitt et al. (2000) listed 182 species

of *Aspergillus* with 24 synonyms and eight holomorphic genera that associated with *Aspergillus* anamorphs. Between 1992 and 1999, Samson (2000) listed another 36 taxa. Since then, more than 40 new species have been described, bringing the total number to approximately 250 species and the number was increasing (Geiser et al., 2007).

Samson et al. (2007) recommended a polyphasic approach to determine correct species name and taxonomical position of *Aspergillus* species. Polyphasic approach combined macro and micromorphological features, growth characters, extrolite profiles, and sequencing data of three or more regions or protein coding genes such as ITS, β-tubulin and calmodulin. This approach provided more information on unresolved species which has been previously described as closely related and having similar morphological characteristics.

With the introduction of one fungus, one name concept, the dual nomenclature of *Aspergillus* which taking into account the teleomorphic and anamorphic genera, is no longer acceptable. As only one fungal species name is required, ICPA has decided to maintain the name *Aspergillus* for all species including those with teleomorph stage (Samson et al., 2014). This decision resulted in the loss of well-known teleomorph genera such as *Emericella, Eurotium* and *Neosartorya*. The used of this new concept has been applied in several studies including Hubka et al. (2013) and Matsuzawa et al. (2014) to describe two new species, *A. waksmanii* and *A. pernambucoensi.*

A comprehensive and standardized methods for the identification and characterization of *Aspergillus* was introduced by Samson et al. (2014). A number of accepted species that lacking an *Aspergillus* name and the recent accepted 339

species of *Aspergillus* species are listed in the publication with information on living ex-type culture collection numbers and GenBank accession numbers for all regions or genes used for identification.

2.2 *Aspergillus* Section *Nigri*

Aspergillus section *Nigri* or commonly known as black *Aspergillus* are members of the *Aspergillus* subgenus *Circumdati,* formerly known as *Aspergillus niger* group. Many taxonomical changes have occurred in *Aspergillus* section *Nigri* particularly on phenotypic and molecular characteristics. Until 2009, there are 18 species of black *Aspergillus* of which several species are rare and mainly found in tropical regions (Nielsen et al., 2009). The number was then increased to 26 species and can be divided into five main clades, *A. niger, A. carbonarius, A. heteromorphus, A. homomorphus* and *A. aculeatus* based on calmodulin gene sequences (Varga et al., 2011). Later, the existence of 26 species of black *Aspergillus* were divided into the same main clades as listed in Table 1 which were confirmed by Samson et al. (2014) using single-nucleotide polymorphisms (SNPs) based on partial calmodulin gene sequences.

Table 2.1: Classification of 26 type strains of black *Aspergillus* using SNPs of partial calmodulin gene sequences

Clades	Species
A. niger	*A. neoniger*
	A. costaricaensis
	A. vadensis
	A. piperis
	A. lunchuensis
	A. tubingensis
	A. awamori
	A. niger
	A. brasiliensis
A. carbonarius	*A. ibericus*
	A. sclerotiicarbonarius
	A. carbonarius
	A. sclerotioniger
A. heteromorphus	*A. eliipticus*
	A. heteromorphus
A. homomorphus	*A. saccharolyticus*
	A. homomorphus
A. aculeatus	*A. fijiensis*
	A. aculeatus
	A. aculeatinus
	A. uvarum
	A. indologenus
	A. japonicus
	A. brunneoviolaceus
	A. floridensis
	A. trinidadensis

Source : Varga et al. (2011); Samson et al. (2014)

The present classification of black *Aspergillus* based on National Centre for Biotechnology Information (NCBI) are as follows:

Kingdom : Fungi

Subkingdom : Dikarya

Phylum : Ascomycota

Subphylum : Pezizomycotina

Class : Eurotiales

Order : Eurotiales

Family : Aspergillaceae

Genus : *Aspergillus*

Subgenus : *Circumdati*

Section : *Nigri*

The taxonomy of black *Aspergillus* is one of the most confusing and complex due to slight morphological and biochemical differences between species. For convenient, some researchers refer to *A. niger* as 'species complex' as several species such as *A. niger* and *A. tubingensis* are morphologically or biochemically similar and thus are indistinguishable (Varga et al., 1993). There are also some of the black *Aspergillus* species which are distinguishable based on morphological characteristics such as *A. carbonarius* and other uniseriate species of black *Aspergillus* (Martinez-Culebras and Ramon, 2007). Thus, in several studies, the term

'*A. niger* aggregate' was used, referring to several black *Aspergillus* species. In order to overcome morphological and biochemical limitations, molecular identification and characterization are applied. However, morphological identification and characterization are still widely applied to separate or to group the isolates into morphological groups.

2.3 Identification and Characterization of Black *Aspergillus*

2.3.1 Morphological Identification

The used of microscopic and macroscopic characteristics as primary tools in the identification of various species of black *Aspergillus* were applied in several manuals and publications such as Raper and Fennell (1965), Klich (2002b), Samson et al. (2007), Pitt and Hocking (2009), Silva et al. (2011), and Samson et al. (2014).

Macroscopic characteristics including colony appearance and texture, colony pigmentation, colony diameters, presence of exudates, sclerotia and cleistothecia are used to differentiate species into sections. Pitt and Hocking (2009) emphasized the used of colony colours for species lacking teleomorph stage as the colours consistently associated with a particular species while Klich (2002) suggested the used of conidial colours as major feature for subgeneric classification. *Aspergillus* section *Nigri* is easily characterized by the black and dark brown colonies formed on most of the culture media used (Klich, 2002b; Samson et al., 2010).

For microscopic characteristics, several features including formation of phialides (uniseriate/biseriate), the shape of vesicle, the size and texture of stipe, and the size and texture of conidia are used for species identification (Figure 2.1). Phialide is a conidiogenous cell that produces conidia without an increase in the

length of the phialide itself (Pitt and Hocking, 2009). The formation of only phialides is called uniseriate while the formation of both phialides and metulae in a species is called biseriate (Figures 2.1 A and B). In the biseriate species, both metulae and phialides always stick together. Black *Aspergillus* consists of species with both uniseriate and biseriate phialides (Klich, 2002b).

Vesicle is the apical swelling of a stipe that formed in various shapes such as globose or spherical, pyriform, spathulate and clavate (Figure 2.2.). Besides the shapes, size of vesicle provided additional information on the classification of *Aspergillus* species. Conidia is an asexually producing spore which formed at the end of phialides in columnar (compact columns) or radiate (diverging). Other characteristics used to identify black *Aspergillus* species are cleistothecia and sclerotia. Cleistothecia are fruiting bodies without a special opening that contain asci and ascospores. Sclerotia are resting bodies which usually globose and consisting of a compacted mass of mycelium that often very hard (Pitt and Hocking, 2009).

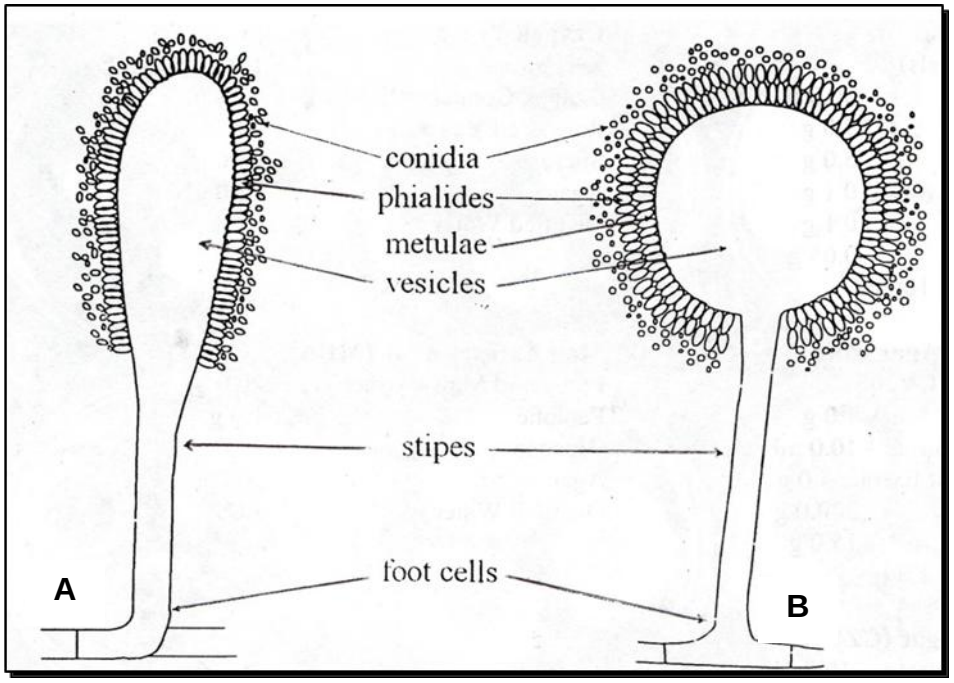

Figure 2.1: Microscopic structures of *Aspergillus* (A) Conidiophore of uniseriate phialide; (B) Conidiophore of biseriate phialide (Klich, 2002b)

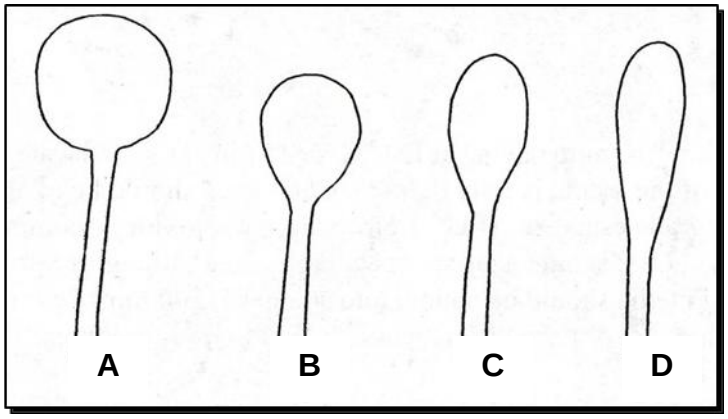

Figure 2.2: Some common vesicle shapes of *Aspergillus* (A) globose or spherical; (B) pyriform; (C) spathulate; (D) clavate (Klich, 2002b)

Morphological identification of black *Aspergillus* species are carried out using differential media. In earlier studies of *Aspergillus* species, Czapek's Solution Agar (CZ) was used by Raper and Thom (1949) and Raper and Fennell (1965) to examine *Aspergillus* cultures under standardized laboratory conditions. As some strains do not grow well on CZ, Czapek Yeast Extract Agar (CYA) was developed by Pitt (1973 and 1979) to improve the growth. Pitt (1973) suggested the use of two basic media that contained inorganic nitrogen source and complex nitrogen which correlated to CYA and Malt Extract Agar (MEA) for identification of *Aspergillus* species.

In addition to CZ and MEA, Klich (2002b) recommended to use CYA and CYA with 20% sucrose (CY20S). All the media are incubated at 25ºC with additional CYA incubated at 37ºC. Pitt and Hocking (2009) and Samson et al. (2010) added two other media, Dicholoran 18% Glycerol Agar (DG18) and Creatine Sucrose Agar (CREA) for characterization of xerophilic *Aspergillus* and acid production by *Aspergillus*. Samson et al. (2010) also suggested to use MEA with 20% sucrose (MEA20S), Yeast Extract Sucrose agar (YES) and Oatmeal Agar (OA) for identification of *Aspergillus* species.

Differential media are useful for morphological identification of black *Aspergillus* as each medium has specific purposes to differentiate certain species within section *Nigri*. For example, growth of xerophilic fungi which are associated with low moisture content can be observed on DG18 (Pitt and Hocking, 2009) while the ability of black *Aspergillus* to produce acid and base can be observed on CREA (Samson et al., 2007). Oat meal agar is use to observe sexual structures of black *Aspergillus* (Samson et al., 2010). Different incubation temperatures are used to

observe the growth and adaptability of black *Aspergillus* species to certain environmental changes.

Morphological identification using differential media is not always reliable as several species of black *Aspergillus* are closely related and produced similar morphological characteristics. For example, *A. carbonarius, A. sclerotioniger* and *A. ibericus* which formed large conidia could not be distinguish based on the growth response on CREA (Samson et al., 2010). In a study by Varga et al. (2011), *A. niger* and *A. awamori* have similar growth rates even at different temperatures, which could not reliably differentiate the two species.

Similar and overlapping morphological characteristics of black *Aspergillus* species may also lead to species misidentification. In several studies, *A. niger* and *A. tubingensis* were assigned as *A. niger* aggregate due to indistinguishable morphological characteristics (Kusters-Van Someren et al., 1991; Accensi et al., 1999; Gonzalez-Salgado et al., 2005). In addition to *A. niger* and *A. tubingensis*, another two black *Aspergillus*, *A. foetidus* and *A. brasiliensis* were also referred as *A. niger* aggregate (Parenicova et al., 2001). Thus, identification solely based on morphological characteristics is inadequate and not always reliable. Due to this limitation, molecular identification and phylogenetic analysis are applied to support the morphological results and to confirm the species identity.

2.3.2 Molecular Identification

Molecular methods are widely used to evaluate genetic relatedness among fungal species and to clarify relationships within or between closely related species.

The sensitivity and specificity of molecular methods used have been resulted in extensive advances in fungal taxonomy (Magnani et al, 2005). For identification of black *Aspergillus,* several molecular techniques including DNA sequencing and PCR-based techniques are useful and reliable in clarifying the taxonomy and identification of the fungal species.

2.3.2(a) DNA Sequencing

ITS region is proposed as primary fungal barcode marker (Schoch et al., 2012) for identification of many fungal species. ITS region comprises ITS1 and ITS2, separated by 5.8S gene and situated between the conserved flanking regions of the small 18 S and large 28 S subunits of ribosomal DNA (Figure 2.3). DNA sequencing of ITS region has been employed as the main targets for identification of black *Aspergillus* as these non-coding regions are highly conserved and can be easily amplified. Moreover, universal ITS primers have designed by White et al. (1990) and the existence and availability of large, easily access ITS dataset in public databases (Balajee et al., 2009).

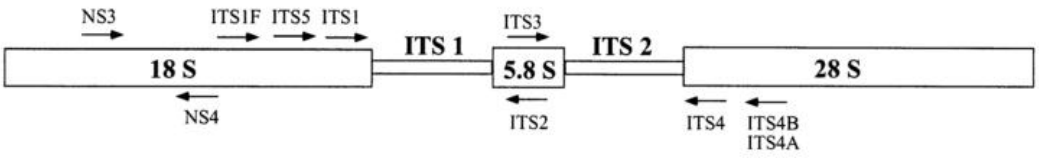

Figure 2.3: Schematic diagram of ITS region with universal ITS primers (ITS1, ITS2, ITS3, ITS4 and ITS5) location (Larena et al., 1999)

The used of ITS region for identification of black *Aspergillus* has been demonstrated by Henry et al. (2000) and Storari et al. (2010). In studies by Medina et al. (2005) and Lasram et al. (2013), ITS region was used to confirm the identity of *A. tubingensis* and *A. carbonarius* isolates. Gonzales-Salgado et al. (2005) also used ITS region in designing species-spesific primers in order to discriminate several black *Aspergillus* species such as *A. japonicus*, *A. heteromorphus*, *A. ellipticus*, *A. niger* and *A. tubingensis.*

Even though ITS region has been used widely in identification of black *Aspergillus* and other *Aspergillus* spp., Balajee et al. (2007) suggested the used of ITS region up to subgenus and sections levels only. Among the limitations of ITS region are having insufficient resolution to differentiate species within *Aspergillus* sections, inability to differentiate closely related species due to lack of nucleotide differences and problems with the reliability of ITS sequences deposited in reference databases (Balajee et al., 2009). The lack of resolution of ITS region at species level was reported by Parenicova et al. (2001), Lasram et al. (2013) and Kamal et al. (2015) in which ITS region was not able to distinguish closely related species of *Aspergillus*.

In order to overcome the limitations associated with ITS region, additional protein coding genes including β-tubulin, α-calmodulin, RNA polymerase II second largest subunit (*RPB2*) and translation elongation factor-alpha (*TEF*) have been used to distinguish closely-related species of black *Aspergillus*. The possibility of using β-tubulin, α-calmodulin or the *RPB2* as alternative markers has been discussed by Samson et al. (2014). Multilocus sequence analysis which is based on β-tubulin, α-calmodulin, *RPB2* and *TEF* were reported by Susca et al. (2013) as practical tools in identifying species of black *Aspergillus*. In general, protein coding genes consist of intron-rich portions which are highly conserved and have variable sequences

(Khoury and Atoui, 2010). Thus, these genes are important for species-level phylogenetic analysis (Geiser et al., 2007) and can be used as alternative markers for identification of black *Aspergillus*.

β-tubulin and α-tubulin are the most common members of the globular proteins which make up microtubules (Dangre et al., 2009). Sequence analysis of partial β-tubulin gene was commonly performed using primer pairs Bt1a and Bt1b, and Bt2a and Bt2b. These primers were originally constructed and designed based on β-tubulin gene of *Neurospora crassa* (Glass and Donaldson, 1995) (Figure 2.4). Balajee et al. (2007) recommended the used of β-tubulin for intra section identification of *Aspergillus*. In a study by Morello et al. (2007), species-specific primers to detect *A. westerdijkiae* from coffee beans were designed based on genetic variation between β-tubulin gene sequences of *A. ochraceus* and *A. westerdijkiae*. According to Samson et al. (2007) all species of black *Aspergillus* can be differentiated using β-tubulin sequences except for *A. lacticoffeatus* that has identical β -tubulin sequences with some *A. niger* isolates.

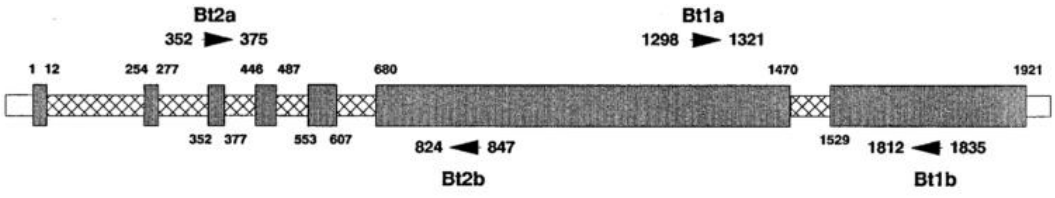

Figure 2.4: Schematic diagram of β-tubulin gene with primers (Bt1a, Bt1b, Bt2a and Bt2b) location (Glass and Donaldson, 1995)

The word calmodulin comes from ‘CALsium MODULated proteIN’ which can be found abundantly in the cytoplasm of all eukaryotic cells (Niessen et al., 2005). Calmodulin gene was recommended by Varga et al. (2011) to be used for identification of black *Aspergillus*. Later, calmodulin gene has also been proposed as secondary marker for identification of *Aspergillus* spp. by Samson et al. (2014). Calmodulin gene consists of several species-specific diagnostic traits. The traits are basically based on the sequence differences of conserved and polymorphic regions related to exons and introns of the calmodulin gene (Susca et al., 2007). One of the calmodulin primers that commonly used for identification of *Aspergillus* are cmd5 and cmd6 designed by Hong et al. (2006). The primer pairs were originally constructed based on complete calmodulin gene of *A. oryzae* sequence with introns and exons 2, 3, 4 and partial exon 5.

In a study by Storari et al. (2012), calmodulin gene amplified using CL1 and CL2 primers were used to identify black *Aspergillus* from herbal teas in Swiss market. Similarly, *A. welwitschiae*, a species of black *Aspergillus* was identified based on calmodulin sequences (Massi et al., 2016). In another studies by Susca et al. (2007) and Kamal et al. (2015), sequence differences of calmodulin gene has been proven to be highly useful to distinguish black *Aspergillus* species, particularly in designing species-specific primers for identification of *A. niger* and *A. tubingensis*. Similar study was also reported by Palumbo et al. (2011) of which species specific primers were designed from calmodulin gene to distinguish *A. niger*, A. *welwitschiae*, *A. carbonarius* and *A. tubingensis*.

The used of more than one region or gene in molecular identification of black *Aspergillus* species was recommended by Samson et al. (2014). DNA sequencing of ITS region should be used as primary marker as this region has been sequenced

repeatedly in fungi thus the primers are universally available. DNA sequencing of calmodulin gene was suggested as temporary secondary marker as the sequence database is nearly complete for all accepted species of *Aspergillus*. Both ITS region and calmodulin gene are recommended to be used for identification while β –tubulin and *RPB2* are recommended for phylogenetic study of *Aspergillus* spp (Samson et al., 2014).

2.3.2(b) PCR-RFLP of ITS

Restriction fragment length polymorphism is based on the variations in the genome of different groups or strains of organism (Narayanasamy, 2011). In addition to genome mapping, RFLP analysis of rDNA was also used in variation analysis which include genotyping. ITS-RFLP consists of PCR amplification of the fungal ITS region, followed by digestion of the PCR product with different types of restriction endonuclease. Polymorphism were observed in the restriction patterns when mutation of a single base-pair occurs and resulted in the loss of a new restriction site or when there is an insertion/deletion, the size of a restriction fragment is altered (Brettschneider, 1998). Thus, analysis of the ITS region using PCR-RFLP is useful in gathering presence or absence of restriction sites in the amplified fragments (Feibelman et al., 1998).

PCR-RFLP was described as inexpensive, easy to design and no requirement for advanced instruments (Rasmussen, 2012). In *Aspergillus*, ITS-RFLP has been used widely to characterize and differentiate closely related species that are difficult to distinguish morphologically. The used of RFLP for *Aspergillus* characterization was introduced by Kusters-Van Someren et al. (1990). In the study, ribosomal

banding patterns and the hybridisation patterns of black *Aspergillus* isolates was analysed using pectin lyase genes as probes for hybridisation. As a result, black *Aspergillus* were separated into *A. japonicus, A. heteromorphus, A. ellipticus, A. carbonarius* and *A. niger* aggregate. Later, Kusters-van Someren et al. (1991) proposed the division of *A. niger* aggregate into two species, namely *A. niger* and *A. tubingensis* according to RFLP analysis.

Accensi et al. (1999) and Parenicova et al. (2001) described the used of ITS-RFLP to differentiate *A. niger* aggregate into two RFLP patterns, type N and type T species which corresponded to *A. niger* and *A. tubingensis*, respectively. *Aspergillus niger, A. tubingensis, A. carbonarius* and *A. aculeatus* isolates were also identified by their ITS-RFLP profiles (Martinez-Culebras and Ramon, 2007; Spadaro et al., 2012). In other studies, ITS-RFLP has been used to characterize black *Aspergillus* at species level (Oliveri et al., 2008; Spadaro et al., 2012; Kizis et al., 2014). In mycotoxin studies, ITS-RFLP have been used to identify ochratoxigenic black *Aspergillus* from cocoa beans (Bisbal et al., 2009), grapes (Zanzotto et al., 2006) and wines (Martinez-Culebras and Ramon, 2007) based on the ITS-RFLP profiles.

Besides RFLP, Random Amplified Polymorhic DNA (RAPD) and Amplified Fragment Length Polymorphisms (AFLP) have been used for characterization of *Aspergillus* species particularly black *Aspergillus* (Megnegneau et al., 1993; Schmidt et al., 2003). Random Amplified Polymorhic DNA has been applied by Ferracin et al. (2009) to characterize *Aspergillus* spp. as well as to identify and to observe genetic diversity of *A. niger* from soil, seeds, powdered milk and factory waste water (Hashoosh et al., 2015). As for AFLP, Perrone et al. (2008) used this technique to characterize black *Aspergillus* species from grapes while Varga et al. (2007) used AFLP to describe *A. brasiliensis* and *A. uvarum*, a novel species of black *Aspergillus*

from soil and grapes. Both AFLP and RAPD analysis need a strict standardisation of conditions and workload thus making these techniques quite difficult to handle.

2.3.3 Phylogenetic Analysis

In phylogenetic analysis, evolutionary relationships among individuals or groups of organisms can be estimated using DNA sequences and illustrated in a phylogenetic tree (Rokas, 2011). Phylogenetic analysis has also been used to show relatedness among the groups of individuals as well as to determine the genetic variation and biodiversity or even ecology of the organisms. In taxonomical studies of *Aspergillus* spp., phylogenetic analysis was found to be useful for identification and characterization thus able to clarify the taxonomical relationships among the species.

Building a phylogenetic tree from molecular data is a simple process that can be learned in a short time (Hall, 2013). The branches of the phylogenetic tree represent the evolutionary relationships among the individuals with neighbouring branches showed the most closely related species (Dangre et al., 2009). In addition, the length and nesting of the branches showed the degree of similarity which is calculated when the sequences are compared (Dangre et al., 2009).

In order to study phylogenetic relationship within subgenera and sections of *Aspergillus* spp., several protein coding genes or multilocus sequences has been applied (Geiser et al., 2007). According to Schoch et al. (2012), the phylogenetic analysis of the large subunit of rDNA is the most widely used marker to show relationships within the genus *Aspergillus*. Thus, the interspecies and intraspecies